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Title should read: "Enhanced Immuno-
logical Protection Against Toxic Agents
Via Transection of Rearranged Immuno-
globulin Genes into Hematopoietic Stem
Cells" per same telecon.

ANNUAL REPORT 1989-90

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I. Introduction

The primary effort of the research conducted during the first year of Contract N00014-89-C-0305 has been directed at the development of anti-idiotypic (anti-id) antibodies against the myeloma protein MOPC-167 (M167). We have developed anti-id antibodies against this phosphocholine (PC) binding myeloma protein because we will be using the rearranged variable region genes (V_H167 & V_L167) from this myeloma cell line, ligated to germ-line λ & κ constant region genes, for transfection into both mouse and human stem cells. Once these genes have been successfully transfected into stem cells and these cells transplanted into SCID mice, one must have anti-id reagents to: 1) follow the development of the cells expressing the transfected gene products; and, 2) activate the B cells expressing the M167 antibody as an antigen-specific receptor on their cell surface. This model system is to serve as a prototype for testing the feasibility of transfecting stem cells with antibody genes whose product is directed against an agent (pathogenic or chemical) which cannot be safely used as an immunogen. (RH)

II. Results

A. Isolation and Characterization of Anti-M167-idiotypic Antibodies

Our first attempts to develop anti-M167-id reagents were carried out by immunizing C57BL/6 mice with the M167 protein. A large number of hybridoma antibodies were produced and tested in ELISA for specificity to M167 (data not shown). None of the more than 20 hybridomas produced bound specifically to M167. Most of these mouse hybridomas produced antibodies which cross reacted with one or more of the other known PC-binding myeloma or hybridoma antibodies. Based on the success of Desaynard et al. (1) in producing Rat-anti-T15-id antibodies, we next immunized rats with the M167 IgA myeloma protein and boosted them with the IgM anti-PC hybridoma protein HPCM27 (2). This IgM antibody expresses the same V_H1 heavy (H) & V_L24 light (L) chain variable regions as M167. The hybridomas produced were first screened for binding to M167 & HPCM2 and to MOPC-104E (IgM, λ) and MOPC-460 (IgA, κ) in order to select only those binding to V-region determinants. Those having V-region specificity were then screened against a large panel of PC-binding antibodies (Table I) and only those binding to antibodies expressing V_L24 light chains were selected for subcloning and further characterization. The profile of binding specificity of four rat-anti-M167-id antibodies is shown in table I. Three of these anti-M167-id antibodies (28-2-7, 28-4-3, & 28-6-20) are cross-reactive anti-ids; thus, they were found to bind three antibodies (PCG1-2, PCG1-3, and PCG1-14) which express a J558 V_H in association with V_L24 . All three of these antibodies bind nitrophenylphosphocholine (NPPC) (3) and thus may share some structural similarities to PC binding antibodies. The 28-2-7 anti-M167-id antibody differs from the other anti-M167-id antibodies in that it recognizes an idiotope which has been lost by somatic mutation from the M511 and HPCG13 V_H1 : V_L24 anti-PC antibodies. The 28-4-3 and 28-6-20 anti-M167-id antibodies exhibit the same binding profile on the panel of proteins tested and may represent identical clones; however, when tested on spleen cells from transgenic mice expressing M167 transgenes, these two antibodies stain slightly different numbers of cells (Figures 1-3), thus, they could be recognizing different idiotopes. Competitive binding will have to be performed to resolve this issue. The anti-M167-id antibody 28-3-13 is clearly

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different from the other cross-reactive anti-id antibodies since it binds only to PC-specific antibodies expressing the $V_{H1}:V_{L24}$ H + L chain combination. This anti-M167-id antibody has further been shown to be binding-site specific in that it blocks the binding of M167 to PC-BSA coated ELISA plates, whereas, the other three anti-M167-id antibodies are not binding site specific. Based on this observation, one would predict that the 28-5-15 antibody will be the most useful in in vivo selection and activation of M167-id⁺, PC-specific B cells. This hypothesis is currently being tested in normal mice.

B. Idiotypic Analysis of M167 Transgenic Mice

As stated above, the anti-M167-id antibodies have been developed so that they can be used in place of antigen in stimulating B lymphocytes which express these idiotopes on their antigen-specific receptors. However, it is critical that the anti-id used for stimulation activate B cells which are indeed antigen-specific and not clones which might share cross-reactive idiotopes but have a different antigen specificity.

To further characterize and evaluate our anti-M167-id antibodies for their potential in recognizing and activating id⁺-antigen specific B cells, we have taken advantage of a set of transgenic mouse lines developed by Storb et al. (4) in which the rearranged M167 H & L chain genes have been expressed individually or in combination. Spleen cells from these mice were stained with a combination of FITC-conjugated anti-IgM and biotin-conjugated anti-M167-id plus phycoerythrin-conjugated Streptavidin. The stained cells were then analyzed using a Coulter 753 Cytofluorograph. In the M167 μ 207-4 line, greater than 96% of the B cells stain with all three of the anti-M167-ids tested (Fig. 1). In this M167 μ transgenic mouse strain, virtually all the B cells are PC-specific (5) and all three anti-M167-id antibodies should induce activation and proliferation of the B cells from these mice. This assumption is currently being tested.

X-ray crystallography analysis of the MOPC-603, PC-binding myeloma protein has shown that most of the contact determining residues for PC reside in the V_{H1} H-chain (6). Thus, in transgenic mice expressing the M167-H-chain only, one might expect to see the association of this H-chain with endogenous light chains to form antibodies which can bind PC. Since there are approximately 100 to 200 different κ -light chain genes in the mouse (7) one would expect to see approximately 1% of the B cells in an M167 μ -transgenic mouse express the V_{L24} dependent M167-ids. However, when spleen cells from these μ -only transgenic mice were stained with the anti-M167-ids (Fig 2), it was surprising to find that ~10% of the spleen cells or almost 20% of the B cells were M167-id⁺. This means that B cells in which the H-chain transgene has associated with an endogenous V_{L24} light chain have expanded to make up 20% of the B cell population instead of the expected 1%. As seen in fig. 2, there are two IgM⁺:id⁺ populations of B cell in the μ -only transgenic mice; one population expresses high levels of idiotope (~5% of spleen cells) and the second population expresses low levels (3 to 5% of spleen cells). The reason for the two distinct M167-id⁺ population is unclear at this time, but the low intensity staining may be due to the co-expression of endogenous H-chains on many of the B cells in these μ -only transgenic mice. Three color flow cytometric analysis will have to be performed in order to resolve this issue. However, when PC-binding of the μ -only spleen cells is tested for in a rosette assay with PC-coupled SRBC, one finds that ~ 3% of the spleen cells are PC-

specific (Table II). This correlates well with the number of bright id⁺ B cells in these mice.

Why are 20% of the B cells in the μ -only transgenic mice M167-id⁺ rather than the expected 1%? It is possible that the M167-id⁺ B cells in the μ -only transgenic mice are being expanded by stimulation with environmental PC-containing antigens. If this is true, one might also expect to see the same expansion of PC-specific, id⁺ B cells in M167 κ -only transgenic mice. These PC-specific B cells should be formed by the association of the V₂₄ light chain transgene product with an endogenous V_{H1} H-chain. However, as shown in fig. 3 (panels B & C), large numbers of cross-reactive M167-id⁺ (28-4-3 and 28-6-20) B cells are detected in M167 κ -only transgenic mice but very few of these B cells are PC-specific (table 2) or express the binding-site-specific anti-M167-id, 28-5-15. Furthermore, less than 1% of these B cells express the V_{H1} H-chain as determined by staining with the T68.3 anti-V_{H1}-id (data not shown). These data clearly demonstrate that an animal can have large numbers of id⁺ B cells present in its lymphoid tissues and yet the B cells may not be antigen specific. Thus, as stated above, it will be very important to use site specific anti-id to focus in on the activation of the desired antigen-specific B cells.

C. Transfection of H and L Chain Genes to Determine the Requirements for Antigen Binding and Idiotype Expression

It is important to try and understand the above observation, i.e. why having a rearranged M167 H-chain transgene expressed in the germ-line of a mouse resulted in large numbers of antigen-specific B cells expressed in its spleen, while the expression of the rearranged M167 L-chain transgene did not result in the selection of antigen-specific, M167-id⁺ B cells.

In the mouse, the antibody response to PC is highly restricted and is comprised of three related families of antibodies called T15, M167-M511, and M603 (8). All three families use the same V_{H1} gene which is recombined most often with the DFL 16.1 D-region gene and the J_{H1} gene (9) (Fig 4). They differ in that three different V_L genes are used, i.e. V₂₂ in the T15 family, V₂₄ in the M167-M511 family, and V₈ in the M603 family (fig 4). However, as shown by Claflin et al. (10) and Feeney et al. (11) the H-chains also differ due to "family-specific substitutions" at the V_H-D_H junction in amino acid residues 95 and 96 (fig 4). Thus, in the M603 family, there is a somatic mutation of the germ-line aspartic acid to an asparagine residue at position 95 (10), and in the M167-M511 family, there is an alanine inserted at position 96 via an alternate splicing of the V_{H1} gene or N-region diversification (11). We hypothesized that these "family-specific substitution" in the V_{H1} gene might result in the subsequent restricted light chain usage in generating PC-specific antibodies. Thus, having an aspartic acid at residue 95 would mean that a PC-specific antibody would be generated only when this T15 form of the V_{H1} H-chain associated with a V₂₂ L-chain, and the substitution of an asparagine at this position would mean that only the V₈ light chain would generate a PC-specific antibody. Furthermore, the formation of the M167-M511 V_{H1} H-chain by insertion of an alanine between the aspartic acid 95 and tryptophane 96 of the T15 sequence would mean that only the V₂₄ light chain would reconstitute PC-binding. This hypothesis was tested by co-transfecting the different forms of the V_{H1} gene with the three family specific L-chain genes into myeloma or hybridoma cell lines. The

antibodies derived from these transfected cell lines were then analyzed for their ability to bind to PC-BSA coated plates and for the presence of family specific idiotopes, and for H & L chain allotype or isotype (Table III). The data shown in table III strongly suggest that the above working hypothesis is correct. When antibodies expressing the H & L chain combinations shown in table III were tested in a direct binding ELISA, only those antibodies with the known H & L family combinations of V_HT15:V_L22 (line 8), V_H167:V_L24 (line 3) and V_H603:V_L8 (line 12) exhibited strong binding to PC-BSA. The antibody from cell line 6, which expressed a V_HT15 H-chain associated with the V_L8 L-chain, exhibited low but detectable binding to PC. It is interesting to note that in many of the H:L combinations, L-chain dependent idiotopes are regenerated in the absence of PC-specificity (lines 2, 9, 10, & 11). For example, T15 idiotopes are generated when V_L22 associates with V_H167 (line 2) or V_H603 (line 10) and M167 idiotopes are generated when V_L24 associates with V_HT15 (line 9) or V_H603 (line 11). In the V_HT15:V_L24 antibody, even the binding-site-specific idiopeptide 28-5-15 is generated in the absence of detectable affinity for PC. The data in table III further demonstrate that all 9 H & L gene combinations tested resulted in IgM antibodies in which the κ -isotype, μ^s -allotype, and V_H1-idiotope markers were expressed.

The above data provide a possible explanation for the earlier observation that the M167 H-chain preferentially associated with the V_L24 light chain in the 243-4 μ -only transgenic mice. Based on these transfection experiments, it is likely that this H-chain generates PC-specificity only in association with the V_L24 L-chain and that this clone of B cells is then expanded by environmental PC-containing antigens.

D. Loss of M167 Idiotype Positive, PC-Specific B Cells in Mice Expressing an X-linked Immunodeficiency.

The anti-M167-id antibodies described in section A above have proven to be very useful in following the development of antigen-specific, idiotypic positive B cells. In a separate project, which was not directly related to this contract, we have analyzed the effect of the X-linked immunodeficiency gene (xid) on the development of antigen-specific B lymphocytes in two different sets of transgenic mice carrying rearranged heavy and light chain genes coding for anti-PC and anti-TNP antibodies, respectively. In the anti-PC transgenic mice, large numbers of TG⁺, PC-specific B cells are detectable in the spleens of mice bearing a normal X-chromosome, while B cells are either absent or reduced more than 90% in TG⁺ xid mice (see fig 1 and table I of attached manuscript). In contrast, the coexpression of the anti-TNP μ/κ transgenes with the xid gene does not lead to a greater decrease in absolute splenic B cells than occurs in TG⁺ mice with a normal X-chromosome; thus, a 60 % reduction occurs in both types of anti-TNP transgenics compared to their respective TG⁺ controls (see table II and fig. 5 in manuscript). To demonstrate that the loss of PC-specific B cells in the $\mu\kappa$ 207-4 anti-PC transgenic mice was due to an Ig-receptor mediated event rather than a failure of xid mice to develop Ly-1⁺ or Lyb-5⁺ B cells, we analyzed B cell development in xid mice expressing a M167 μ H-chain only. The data presented in figure 4 and in tables III & IV of the accompanying manuscript show that: 1) the V_H1- μ^s transgene product is readily expressed in xid B cells, and therefore, it is not developmentally restricted to Ly-1⁺ or Lyb-5⁺ B cells; 2) M167-id⁺ PC-specific B cells are clonally deleted in (B6.CBA/N x 243-4)F1 xid male transgenic mice, whereas, these PC-specific B cells are

greatly expanded in the peripheral lymphoid organs of F1 female μ -transgenic mice; 3) the M167 μ H-chain transgene product appears to associate preferentially with an endogenous V₂₄ light chain to produce large numbers of M167-id⁺ B cells in the spleens of normal TG⁺ F1 female mice; 4) the M167-id is restricted to B cells bearing the transgene-encoded μ^2 -allotype and is not found on B cells expressing only the endogenous μ^2 -allotype, thus, there is no network induced selection of endogenous V_H-idiotypes; and, 5) T15-id⁺ B cells (V_H1:V₂₂), which normally dominate the PC repertoire, appear to be absent in these M167 μ transgenic mice in spite of the fact that a rearranged V_H1 transgene is expressed in these mice. These observations are consistent with the hypothesis that most PC-specific B cells are clonally deleted via an Ig-receptor mediated mechanism following their development in the bone marrow of mice expressing the *xid* gene. The mechanism responsible for this Ig-receptor-directed clonal deletion of PC-specific B cells does not cause a similar elimination of TNP-specific B cells in the Sp6 (μ/κ) anti-TNP *xid* transgenic mice.

The majority of PC-specific B cells in M167 μ TG⁺ *xid* mice appear to be clonally deleted following receptor expression in the bone marrow but before migration to the spleen. However, approximately 2×10^6 B cells are present in the *xid* TG⁺ spleens and these B cells clearly express the μ^2 transgene product (Fig. 2, attached manuscript) but escape clonal deletion. It was possible that these B cells expressed the constant region allotype marker of the transgene but had lost or somatically mutated the V_H or V_L genes so that they neither bound PC nor expressed the M167 or V_H1-idiotypes. To test this possibility, the spleen cells of these μ anti-PC TG⁺ mice were stained with FITC-anti- μ plus biotin conjugated anti-idiotypic antibodies as shown in figure 4 (attached manuscript). These data clearly demonstrate that the majority of these *xid* B cells express both the V_H1-idiotope (panel A) and M167-idiotypes (panels C & D). The 28-5-15 anti-M167-idiotype is dependent on the expression of both the V_H1 H-chain and the V₂₄ L-chain and is binding site specific, whereas the 28-6-20 anti-M167-idiotope recognizes V₂₄ in association with additional V_H gene products and is not binding site specific (Sieckmann et al. manuscript in preparation). There is a minor population of B cells (17 to 36%) in the TG⁺ *xid* mice which is not recognized by these anti-M167-idiotypic antibodies but clearly bear the V_H1-idiotope. The number of PC-specific antigen binding cells (ABC) is approximately equivalent to the number of M167-idiotype-positive B cells present in the μ TG⁺ *xid* mice (data not shown). These data demonstrate that a subset of M167-id⁺ B cells is escaping clonal deletion in the *xid* mice, and this point would have been impossible to demonstrate without the anti-id reagents which we developed for the Navy contract related work.

Statement "A" per telecon Capt. Stephen Lewis. Naval Medical Research & Development Command/code 405. Bethesda, MD 20814-5044.

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Table I

BINDING SPECIFICITY OF RAT ANTI-M167-IDIOTYPIC ANTIBODIES

| | | | Rat anti-M167-id | | | |
|--------------------------------|------------------------------------|---------|----------------------------------|--------|---------|---------|
| Myeloma/Hybridoma ^a | | | 28-2-7 | 28-4-3 | 28-5-15 | 28-6-20 |
| Name | H/L Chain | Isotype | O.D. ^b ₄₀₅ | | | |
| M167 | V _H 1/V _L 24 | IgA | >2 | >2 | >2 | >2 |
| M511 | V _H 1/V _L 24 | IgA | 0 | >2 | >2 | >2 |
| M27 | V _H 1/V _L 24 | IgM | >2 | >2 | >2 | >2 |
| HPCG13 | V _H 1/V _L 24 | IgG1 | 0 | >2 | 1.2 | >2 |
| HPCG23 | V _H 1/V _L 24 | IgG1 | >2 | >2 | 0.3 | >2 |
| HPCG24 | V _H 1/V _L 24 | IgG1 | >2 | >2 | 0.5 | >2 |
| HPCG28 | V _H 1/V _L 24 | IgG1 | >2 | >2 | 1.5 | >2 |
| PCG1-2 | J558/V _L 24 | IgG1 | >2 | >2 | 0 | >2 |
| PCG1-3 | J558/V _L 24 | IgG1 | >2 | 0.2 | 0.1 | 0.5 |
| PCG1-14 | J558/V _L 24 | IgG1 | >2 | >2 | 0 | >2 |
| HPCM2 | V _H 1/V _L 22 | IgM | 0 | 0 | 0 | 0 |
| H8 | V _H 1/V _L 22 | IgA | 0 | 0.2 | 0 | 0.1 |
| T15 | V _H 1/V _L 22 | IgA | 0 | 0 | 0 | 0 |
| CEBP3 | V _H 1/V _L 22 | IgA | 0 | 0 | 0 | 0 |
| HPCG11 | V _H 1/V _L 22 | IgG3 | 0 | 0 | 0 | 0 |
| F59-GC5 | V _H 1/V _L 23 | IgG3 | 0 | 0.1 | 0.1 | 0.1 |
| 103.1C9 | V _H 1/V _L 22 | IgG2b | 0 | 0 | 0 | 0 |
| PCG2a-2 | V _H 1/V _L 22 | IgG2a | 0 | 0 | 0 | 0 |
| M603 | V _H 1/V _L 8 | IgA | 0 | 0 | 0 | 0 |
| 1B8E5 | V _H 1/V _L 1 | IgG2b | 0 | 0 | 0 | 0 |
| PCG2b-3 | Q52/V _L 1 | IgG2b | 0 | 0 | 0 | 0 |
| PCG3-3 | Q52/V _L 1 | IgG3 | 0 | 0 | 0 | 0 |
| T183 | | IgM | 0 | 0 | 0 | 0 |
| M104E | J558/V _L 1 | IgM | 0.1 | 0.1 | 0 | 0 |
| M315 | J558/V _L 2 | IgA | 0 | 0 | 0 | 0 |
| M460 | J558/V _L 21 | IgA | 0 | 0 | 0 | 0.1 |

- a) Purified myeloma or hybridoma proteins were coated onto microtiter plates using 100 μ l of a 5 μ g/ml solution in phosphate buffered saline pH 7.4.
- b) Tissue culture supernatants of the rat anti-M167-id hybridomas were titrated on the coated plates and the plates developed with a biotin conjugated anti-rat- κ (MAR 18.5) or goat-anti-rat IgG. The O.D. at a 1:10 dilution of the hybridoma supernatant is shown above.

Table II

Development of Antigen-Binding Cells in M167 Transgenic Mice^a

| Strain | Transgenes | Phenotype | % ABC |
|--------|-----------------------|----------------|-------|
| 207-4 | $\mu\kappa$ | T ⁺ | 46.0 |
| | | T ⁻ | 0.3 |
| 216-7 | $\mu\kappa\Delta mem$ | T ⁺ | 0.2 |
| | | T ⁻ | 0.02 |
| 243-4 | μ | T ⁺ | 5.05 |
| | | T ⁻ | 0.05 |
| 254-3 | $\mu\Delta mem$ | T ⁺ | 0.02 |
| | | T ⁻ | 0.02 |
| 234-4 | κ | T ⁺ | 0.05 |
| | | T ⁻ | 0.05 |

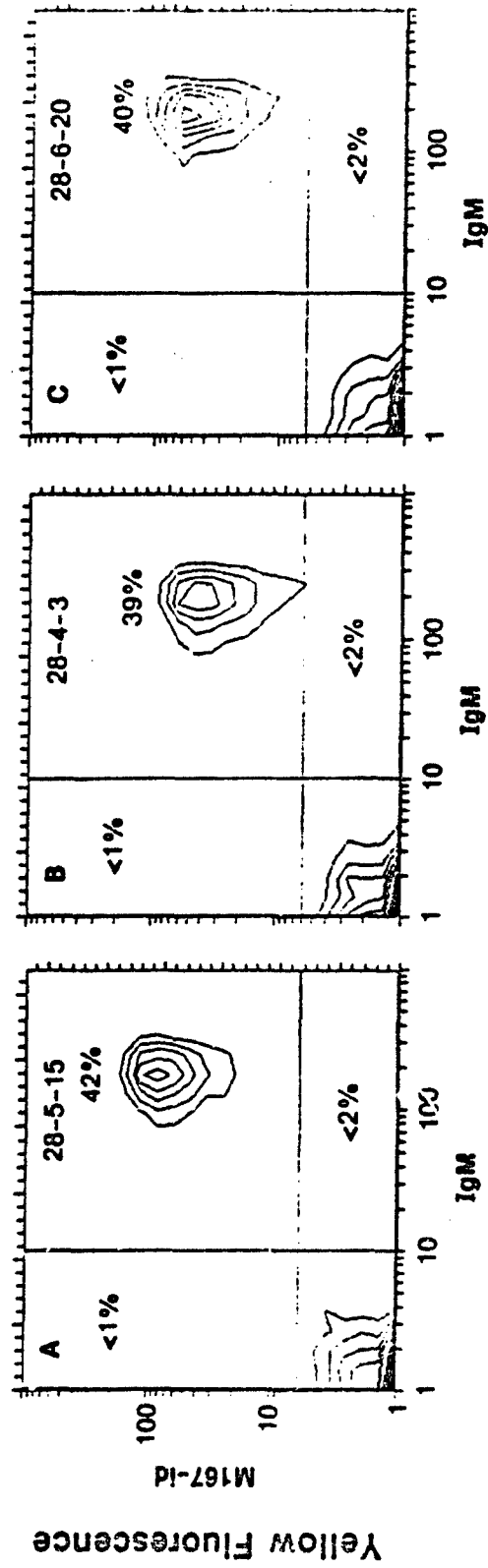
a) Two hundred μ l of spleen cells (1×10^7 /ml) from each of the above M167 transgenic mouse strains was mixed with 100 μ l of 2% v/v PC-conjugated sheep red blood cells (PC-SRBC). The cells were spun at $-800 \times g$ for 10 min at 4°C, and the rosetted cells gently resuspended, placed on a Hemocytometer and the antigen binding cells (ABC) counted.

Table III

Characterization of Cell Lines Transfected with PC-Binding H and L Chain Genes

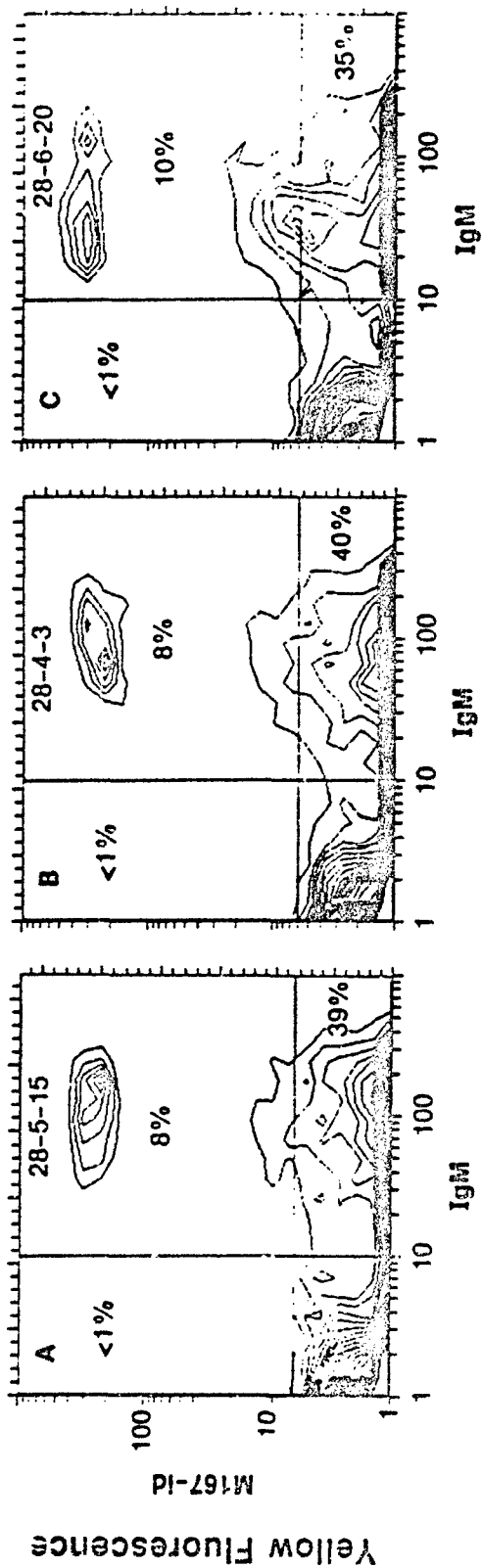
| Cell Transfected Line V-genes | Kappa | Lambda | IgM ^a | V _H 1-id | T15-id | | M167-id | | PC-BSA |
|--|-------|--------|------------------|---------------------|--------|-------|---------|--------|--------|
| | | | | | T139.2 | AB1.2 | 28-5-15 | 28-4-3 | |
| 1. J558L + V167 μ | - | + | + | + | - | - | - | - | - |
| 2. J558L + V167 μ + V _K 22 | + | + | + | + | + | + | - | - | - |
| 3. J558L + V167 μ + V _K 24 | + | + | + | + | - | - | + | + | + |
| 4. 301.2D7.7 V _K 8 | - | - | - | - | - | - | - | - | - |
| 5. 301.2D7.7 V _K 8 + V167 μ | + | - | + | + | - | - | - | - | - |
| 6. 301.2D7 V _K 8 + VT15 | + | - | + | + | - | - | - | .. | +/- |
| 7. SP2.0 | - | - | - | - | - | - | - | - | - |
| 8. SP2.0 + VT15 μ + V _K 22 | + | - | + | + | + | + | - | - | + |
| 9. SP2.0 + VT15 μ + V _K 24 | + | - | + | + | +/- | +/- | + | + | - |
| 10. SP2.0 + VM603 μ + V _K 22 | + | - | + | + | + | + | - | - | - |
| 11. SP2.0 + VM603 μ + V _K 24 | + | - | + | + | - | - | - | + | - |
| 12. 301.2D7.7 V _K 8 + VM603 | + | - | + | + | - | - | - | - | + |

pk 207-4 Spleen Cells



Green Fluorescence

μ 243-4 Spleen Cells



Green Fluorescence

K 234-4 Spleen Cells

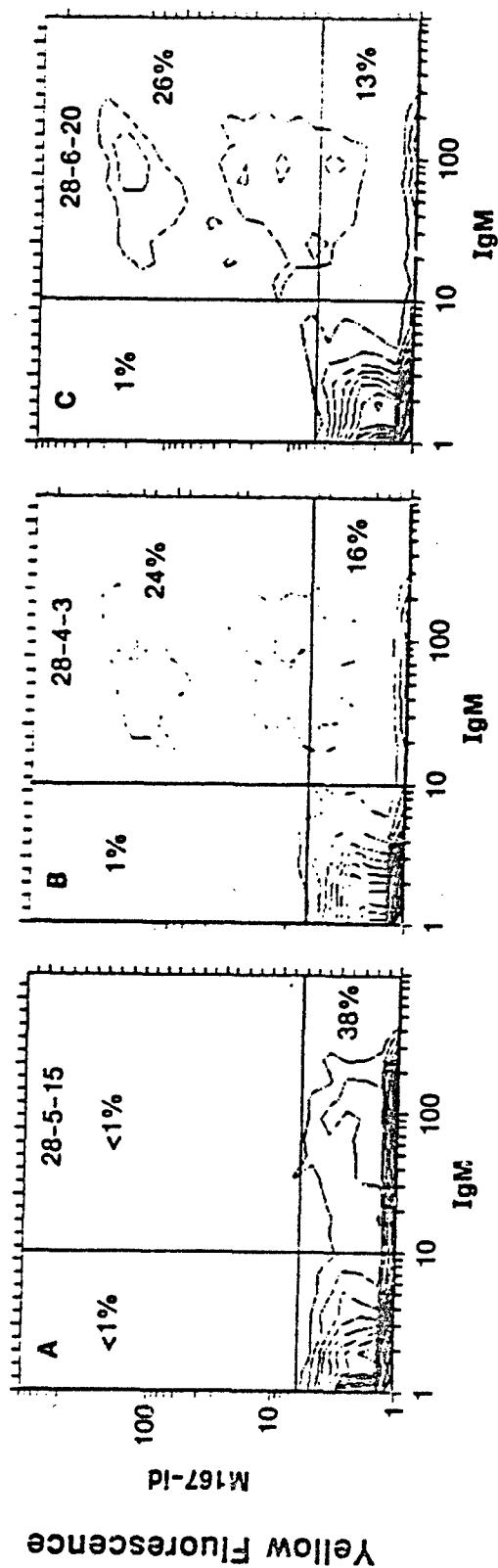


Figure 4

| V _H -1 | | DFL16.1 | | J _H 1 | |
|---|-------------------------|--|---------------------------------|---------------------------------|--|
| 91 | Tyr Cys Ala Arg Asp Ala | Tyr Tyr Gly Ser Ser Tyr | Tyr Tyr Gly Ser Ser Tyr | Tyr Tyr Phe Asp Val | |
| 92 | TAC TGT GCA AGA TAT GCA | TAT TAC GGT AGT AGC TAC | CACAGTG Tyr Trp Tyr Phe Asp Val | CACAGTG TAC TGG TAC TTC GAT GTC | |
| 93 | | | | | |
| 94 | | | | | |
| 95 | | | | | |
| T15-1d (V _H 1, V _H 22) | | Tyr Cys Ala Arg Asp Tyr Tyr Gly Ser Ser Tyr Tyr Trp Tyr Phe Asp Val | | | |
| H167-1d (V _H 1, V _H 24) | | Tyr Cys Ala Arg Asp Ala Tyr Tyr Gly Ser Ser --- Tyr Phe Asp Val | | | |
| H603-1d (V _H 1, V _H 8) | | Tyr Cys Ala Arg ASH Tyr Tyr Gly Ser Ser Tyr Tyr Trp Tyr Phe Asp Val | | | |

The nucleic acid sequence at the top represents the germ-line sequence for the 5' end of V_H1 gene, the DFL16.1 diversity gene segment, and the J_H1 joining segment. Alternate splicing, N-region diversification or somatic mutation of the T15 germ-line sequence give rise to the H167 or H603 V_H1 protein sequences shown in the bottom part of this figure.